Abdulhadi Bima, Angela Pezic, Cong Sun, Fergus J. Cameron, Christine Rodda, Ingrid van der Mei, Rachel Chiaroni-Clarke, Terence Dwyer, Andrew Kemp, Jun Qu, John Carlin, Justine A. Ellis and Anne-Louise Ponsonby*

# Environmental and genetic determinants of two vitamin D metabolites in healthy Australian children

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**Abstract**

**Background:** Vitamin D deficiency has been associated with adverse health outcomes. We examined genetic and environmental determinants of serum $25(OH)D_3$ and $1,25(OH)_2D_3$ in childhood.

**Methods:** The study sample consisted of 322 healthy Australian children (predominantly Caucasians) who provided a venous blood sample. A parental interview was conducted and skin phototype and anthropometry measures were assessed. Concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ were measured by selective solid-phase extraction-capillary liquid chromatography-tandem mass spectrometry. These concentrations were deseasonalised where relevant to remove the effect of month of sampling.

**Results:** Deseasonalised log $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations were only moderately correlated ($r = 0.42$, $p < 0.001$). The following predicted both $25(OH)D_3$ and $1,25(OH)_2D_3$: UVR 6 weeks before the interview, natural skin and eye colour, height and vitamin D allelic metabolism score. The following predicted $25(OH)D_3$ only: lifetime sunburns and vitamin D allelic synthesis score. Overall, 43.5% and 25.6% of variation in $25(OH)D_3$ and $1,25(OH)_2D_3$ could be explained. After accounting for $25(OH)D_3$ concentrations, higher UVR 6 weeks before the interview and vitamin D allelic metabolism score further predicted $1,25(OH)_2D_3$ concentrations.

**Conclusions:** Environmental factors and genetic factors contributed to both vitamin D metabolite concentrations. The intriguing finding that the higher ambient UVR contributed to higher $1,25(OH)_2D_3$ after accounting for $25(OH)D_3$ concentrations requires further evaluation.

**Keywords:** 1,25-dihydroxyvitamin $D_3$; allelic score; determinants; ultraviolet radiation; vitamin D 25-hydroxyvitamin $D_3$.

***Corresponding author: Anne-Louise Ponsonby,** University of Melbourne, Murdoch Childrens Research Institute, Royal Children's Hospital, Flemington Rd, Parkville 3052, Victoria, Australia, Phone: +61383416372, Fax: +613 93481391, E-mail: anne-louise.ponsonby@mcri.edu.au; and Department of Paediatrics, University of Melbourne, Melbourne, Victoria, Australia
**Abdulhadi Bima:** Murdoch Childrens Research Institute, Melbourne, Victoria, Australia; Department of Paediatrics, University of Melbourne, Melbourne, Victoria, Australia; and Clinical Chemistry Department, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
**Angela Pezic, Cong Sun, Fergus J. Cameron and Rachel Chiaroni-Clarke:** Murdoch Childrens Research Institute, Melbourne, Victoria, Australia; and Department of Paediatrics, University of Melbourne, Melbourne, Victoria, Australia
**Christine Rodda:** Department of Endocrinology, Sunshine Hospital, University of Melbourne, Melbourne, Victoria, Australia
**Ingrid van der Mei:** Menzies Research Institute, University of Tasmania, Hobart, Tasmania, Australia
**Terence Dwyer:** Menzies Research Institute, University of Tasmania, Hobart, Tasmania, Australia; and The George Institute, Institute for Global Health, Oxford, UK
**Andrew Kemp and John Carlin:** Murdoch Childrens Research Institute, Melbourne, Victoria, Australia
**Jun Qu:** Department of Pharmaceutical Sciences, University at Buffalo, State University of New York, Amherst, NY, USA
**Justine A. Ellis:** Murdoch Childrens Research Institute, Melbourne, Victoria, Australia; Department of Paediatrics, University of Melbourne, Melbourne, Victoria, Australia; and Centre of Social and Early Emotional Development, School of Psychology, Faculty of Health, Deakin University, Geelong, Victoria, Australia

## Introduction

Vitamin D deficiency, serum level less than 20 ng/mL (50 nmol/L) or insufficiency, serum level 20 to 29 ng/mL (50–75 nmol/L) is common with an estimated 1 billion people having either condition worldwide [1]. In Australia about 30% of adults have vitamin D insufficiency [2] and a recent report showed that over 16% of Australian infants were vitamin D insufficient [3]. In addition to its metabolic role in skeletal health and calcium homeostasis, vitamin D insufficiency may be associated with an increased risk of several other adverse health outcomes such as multiple sclerosis [4], cancer, type 1 and type 2 diabetes [5].

Vitamin $D_3$ is synthesised in the epidermis from 7-dehydrocholesterol (7-DHC) in a reaction catalysed by ultraviolet B (UVB). About 85%–90% of vitamin D is carried in blood circulation by the vitamin D binding protein (VDBP) which has common genetic polymorphisms that produce variant proteins that differ in their affinity for vitamin D and affect its bioavailable levels [6]. New polyclonal and proteomic methods to measure vitamin D-binding protein will enhance our ability to understand its biological role [7]. Vitamin $D_3$ is then rapidly hydroxylated in the liver to 25-hydroxyvitamin $D_3$, then hydroxylated into its biologically active form, 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) mainly in the kidneys. While the latter is the biologically active form, it has extremely low circulating levels, (partly related to a relatively short circulating half-life) resulting in challenging analytical detectability, using most currently available assays. Furthermore, whilst 25 hydroxylases are relatively unregulated, renal 1 α-hydroxylase is tightly regulated in an endocrine fashion, by hormones including parathyroid hormone and fibroblast growth factor 23 (FGF23). Consequently, $25(OH)D_3$ is the current recommended biomarker for vitamin D status [8] as it is the substrate for conversion to the active $1,25(OH)_2D_3$ and its concentration reflects hepatic vitamin D stores.

Many studies have shown that environmental factors such as sun exposure, life style factors [9], ultraviolet radiation (UVR) [10] and skin pigmentation [11] are associated with $25(OH)D_3$ concentrations. Genetic factors also influence circulating vitamin D concentrations [12–16]. Genome-wide association studies (GWAS) have shown that multiple single nucleotide polymorphisms (SNPs) located at or near vitamin D metabolic pathway genes, such as 24-hydoxylase (*CYP24A1*) and vitamin D binding protein (*GC*) are associated with serum $25(OH)D_3$ concentrations [14–16], accounting for 1%–4% of its overall variance [16].

Few studies have comprehensively examined both environmental and genetic factors in parallel. Previously we reported that sun exposure-related factors account for 36%, genetic factors account for 14% and skin phenotypes accounts for 3.5% of $25(OH)D_3$ variation in an Australian adult population [17]. In a separate study of Australian infants, we found that ambient UVR levels 6 weeks before blood collection can predict 10% of $25(OH)D_3$ variation [3]. In addition, past studies have focused only on $25(OH)D_3$ ; however, new advances in the measurements of $1,25(OH)_2D_3$ now provide the opportunity to investigate the determinants of this metabolite. No study has examined the genetic and environmental factors of both $25(OH)D_3$ and $1,25(OH)_2D_3$ simultaneously in children. In this current study we examined both environmental and genetic determinants of two major vitamin D-circulating metabolites, $25(OH)D_3$ and $1,25(OH)_2D_3$, in a population of healthy children in Melbourne, Australia.

# Materials and methods

## Participants

This study is based on a sample of 322 unselected control children from the Early Environment and Type 1 Diabetes Prevention Project. They were all born and residing in Victoria, and were recruited between March 2008 and November 2011 at the Royal Children's Hospital Day Surgery Unit Melbourne, Australia (latitude 38° South, 145° East).

Children under 18 years admitted for a 1-day surgical procedure, such as plastic surgery, eye surgery, circumcision, hernia repair and others, were eligible to participate in the study. Children who had severe congenital abnormalities, prolonged physical or mental illness, which may affect their activity such as playing, walking, or attending normal school, were excluded. The median age at recruitment was 8.42 years (IQR 6.26–11.06 years) and they were divided into three age groups <5, 5–10 and >10 years (see Table 1), 55% were males and the majority (62.4%) were Caucasians.

Children and their guardians (usually parents) were interviewed by trained research nurses who conducted clinical examinations and helped parents to complete the questionnaires on the day of admission. The questionnaires collected information on direct sun exposure behaviours of both children and parents, including how much time they usually spent in the sun during summer and winter, on weekends and weekdays, and freckling by the end of summer using previously validated instruments [18].

Ethics approval was obtained from the Human Research Ethics Committee of the Royal Children's Hospital, Melbourne, Australia. Informed, written consent for the study was obtained from parents/guardians, and from children 12 years of age and older.

## Examination data

The research nurses conducted the examination and noted the natural skin, hair and eye colour using standardized coloured photographs which we have previously shown to explain 39.7% ($p < 0.001$) of the variation in constitutive skin pigmentation measured by spectrophotometer [19]. Child ethnicity (Caucasian, Asian, African, Aboriginal or Torres Strait Islander, Middle Eastern or other) was determined by parental report. The number of naevi on the left arm was counted using a standardised protocol [20]. Height and weight was measured and body mass index [BMI = weight (kg)/height ($m^2$)] was calculated. Gender- and age-specific BMI cut off points were used to classify children as normal, overweight or obese [21].

Monthly average of the total daily ambient UVR dose was obtained in Standard Erythemal Dose units for Melbourne from 2008 to 2011 [22]. The interview months' UVR was calculated as the average of daily total UVR assessed on the year and month of the interview. Likewise, the UVR for 1 month, 6 weeks and 2 months before the interview were determined [19].

**Table 1:** Child characteristics by serum 25-hydroxyvitamin $D_3$ [25(OH)$D_3$] and 1,25 dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$] concentrations.

| Factor | n (%) | $Log_e$ deseasonalised 25(OH)$D_3$, mean[a] (SD) | $Log_e$ deseasonalised 1,25$(OH)_2D_3$, mean[b] (SD) |
|---|---|---|---|
| Age, years | | | |
| (0–4.9) | 42 (13.0%) | 2.74 (0.39) | 3.60 (0.48) |
| (5–9.9) | 172 (53.4%) | 2.83 (0.38) | 3.69 (0.6) |
| (10–16.9) | 108 (33.5%) | 2.92 (0.35) | 3.86 (0.61) |
| Gender | | | |
| Male | 177 (55.0%) | 2.84 (0.36) | 3.70 (0.61) |
| Female | 145 (45.0%) | 2.86 (0.39) | 3.78 (0.58) |
| Season of blood collection | | | |
| Summer | 38 (11.8%) | 3.08 (0.27)[c] | 4.00 (0.69)[c] |
| Autumn | 80 (24.8%) | 2.9 (0.37)[c] | 3.85 (0.62)[c] |
| Winter | 113 (35.1%) | 2.62 (0.41)[c] | 3.33 (0.56)[c] |
| Spring | 91 (28.3%) | 2.78 (0.42)[c] | 3.69 (0.64)[c] |
| Ethnicity | | | |
| Caucasian | 201 (62.4%) | 2.93 (0.3) | 3.76 (0.56) |
| Non-Caucasian | 64 (19.9%) | 2.62 (0.46) | 3.64 (0.59) |
| Missing | 57 (17.7%) | | |
| Body mass index (BMI) category | | | |
| Normal | 166 (51.6%) | 2.90 (0.33) | 3.76 (0.58) |
| Overweight | 44 (13.7%) | 2.70 (0.47) | 3.66 (0.57) |
| Obese | 45 (14.0%) | 2.77 (0.37) | 3.7 (0.6) |
| Missing | 67 (20.8%) | | |
| Eye colour | | | |
| Brown | 140 (43.5%) | 2.74 (0.39) | |
| Hazel | 38 (11.8%) | 2.86 (0.38) | 3.83 (0.71) |
| Green | 22 (06.8%) | 3.05 (0.26) | 3.79 (0.47) |
| Blue/grey | 68 (21.1%) | 3.00 (0.31) | 3.85 (0.63) |
| Missing | 54 (16.8%) | | |
| Natural skin colour (on examination) | | | |
| Dark | 13 (4.0%) | 2.28 (0.35) | 3.57 (0.37) |
| Olive | 47 (14.6%) | 2.76 (0.47) | 3.67 (0.65) |
| Olive/medium | 75 (23.3%) | 2.83 (0.32) | 3.73 (0.56) |
| Medium/fair | 90 (28.0%) | 2.93 (0.29) | 3.76 (0.58) |
| Fair | 43 (13.4%) | 3.00 (0.29) | 3.81 (0.55) |
| Missing | 54 (16.8%) | | |

[a]Mean of the natural-log-transformed deseasonalised 25(OH)$D_3$ concentration. [b]Mean of the natural-log-transformed deseasonalised 1,25$(OH)_2D_3$ concentration. [c]The mean was obtained from the natural-log-transformed non-deseasonalised 25(OH)$D_3$ and 1,25$(OH)_2D_3$.

## Laboratory data

All 322 children provided a peripheral blood sample, which was separated to isolate plasma from the cellular component within 2 h of collection. DNA was extracted from the white blood cell component using the Qiagen Flexigene Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Up to 10 mL peripheral blood samples were collected in EDTA tubes for each child prior to anaesthesia which were centrifuged within 2 h. Plasma and plasma-depleted whole blood were stored at –70°C until analysis. Plasma 25(OH)$D_3$ and 1,25$(OH)_2D_3$ were measured with selective solid-phase extraction capillary liquid chromatography-tandem mass spectrometry at the Qu Lab, University at Buffalo, New York. This central laboratory has achieved a limit of quantification of 5.04 pg/mL (12 pmol/L) and 0.1 ng/mL (0.25 nmol/L), interday accuracy of 9.5%–13.3% and 2.7%–14% and interday precision of 6.7%–16.6% and 3.1%–8.0% for 1,25$(OH)_2D_3$ and 25(OH)$D_3$, respectively [23]. In DEQAS report the deviations were all <18.4% from two batches of samples. The nine-point calibration curve showed excellent linearity with correlation coefficient greater than 0.99 for both 25(OH)$D_3$ and 1,25$(OH)_2D_3$. The method separated the two targeted metabolites from other vitamin $D_3$ metabolites such as 25(OH)$D_3$ epimer and 24,25dihydroxyvitamin $D_3$ [24]. Thus, the possible interfering effect of these metabolites in 25(OH)$D_3$ and 1,25$(OH)_2D_3$ measurements has been removed.

Based on the evidence of their association with circulating vitamin D from past genetic association studies, we selected 12 SNPs located at or near genes related to the vitamin D metabolic pathway (*CYP2R1* (rs10741657), *DHCR7* (rs12785878), *CYP24A1* (rs6013897, rs2181874, rs2762941, rs4809960), *GC* (rs4588, rs2282679) *VDR* (rs11168275, rs4760655), and skin pigmentation genes (rs8045560, rs3936623) [16, 25, 26]. Genotyping was performed using the Sequenom Mass Array system, of which a detailed description of the assay has been published elsewhere [27]. Sequenom iPlex chemistry was

used according to manufacturer instructions. Cluster plots for all 'non-conservative' genotype calls were manually inspected and re-genotyping was done if necessary. After the exclusion of samples not meeting the quality control threshold (departure from the Hardy-Weinberg equilibrium $p < 0.01$ and <90% call rate), genotype data for 311 participants were generated.

### Allele scores and SNPs selection

We constructed two vitamin D allele scores, synthesis score (Synscore) and metabolic score (Metscore), using four out of the 12 examined vitamin D-related SNPs. *CYP2R1* (rs10741657), *DHCR7* (rs12785878), *CYP24A1* (rs6013897) and *GC* (rs2282679), were selected based on findings from the GWAS of the SUNLIGHT Consortium and our univariate results (data not shown) [16, 28]. *DHCR7* (rs12785878) and *CYP2R1* (rs10741657) are in genes located upstream, and *CYP24A1* (rs6013897) and *GC* (rs2282679) are in genes located downstream of $25(OH)D_3$ production (metabolism). Synscore is the sum of the vitamin D-increasing alleles in the upstream genes (*CYP2R1* and *DHCR7* with a score range 0–4) and Metscore is the sum of vitamin D-increasing alleles in the gene located downstream (*GC* and *CYP24A1* with a score range 0–4) of $25(OH)D_3$ [29].

These allelic scores can be used as instrumental variables for $25(OH)D_3$ and $1,25(OH)_2D_3$ concentration when testing, in Mendelian randomisation analysis, for likely causal association as it represents variants with direct effect on substrate availability for vitamin D metabolites' synthesis.

### Statistical analysis

In the univariate analysis, linear regression models were used to examine the environmental, and genetic determinants of $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations. The distribution of both vitamin D metabolites was skewed and thus log-transformed prior to analysis to improve normality and allow parametric statistical analysis. Log-scale summary values were presented and regression coefficients were interpreted as percent change in outcome per 1 unit change in exposure variable (% change = $1 - \exp(\beta)$ for negative $\beta$ or $\exp(\beta)$-1 for positive $\beta$). As $25(OH)D_3$ and $1,25(OH)_2D_3$ had very different blood levels and units (measured in nmol/L and pmol/L for $25(OH)D_3$ and $1,25(OH)_2D_3$, respectively), the log values and their relative change allowed easier comparison and interpretation of the determinants' effect on these metabolites.

For models not including UVR as a potential confounder, deseasonalised vitamin D concentrations were used as the outcome measure to correct for seasonal variation. We calculated deseasonalised $25(OH)D_3$ and $1,25(OH)_2D_3$ by removing the seasonal variation. We fitted sinusoidal model to data on $25(OH)D_3$ and $1,25(OH)_2D_3$ levels and month (t) of sample collection to model seasonal variation [30, 31]:

$$\text{Concentration level } 25(OH)D_3 \text{ or } 1,25(OH)_2D_3 = \beta_0 + \beta_1 \sin(2\pi/12) + \beta_2 \cos(2\pi/12)$$

The amplitude was calculated using the formula $\sqrt{(\beta_1^2 + \beta_2^2)}$. This predicted value was subtracted from the actual value and added to the overall mean. For some analyses it is of interest to remove the natural seasonal fluctuation in vitamin D levels by deseasonalisation which has been used in previous studies [30, 31].

To construct the final predictive model, we used forward stepwise regression starting with one variable in the model, testing the addition of each variable, based on its R-square from the univariate analysis and its impact on model improvement, until there was no improvement of the model using the likelihood ratio test. Interaction between predictors for both $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations in the final models was assessed by the P-value associated with the Wald test of a product term for the relevant factors in categorical terms.

We used Stata version 14 (StataCorp, College Station, TX, USA) for all analyses.

## Results

Demographic and anthropometric mesures of the participants (n = 322) with the corresponding mean of natural-log-transformed serum $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations are presented in Table 1. Serum $25(OH)D_3$ concentrations was only moderately correlated with $1,25(OH)_2D_3$ concentrations with or without deseasonalisation (Figure 1). There was seasonal variation for both $25(OH)D_3$ and $1,25(OH_2D)_3$ concentrations; participants sampled in summer months (December to February) had higher concentrations of both metabolites, compared with those sampled in winter months (June to August) (Figure 2). We examined how $25(OH)D_3$ concentrations predicted $1,25(OH)_2D_3$ concentrations and found that using $25(OH)D_3$ categories in logistic regression did not fit the data better than a simple linear regression model ($p = 1.00$).

Higher ambient UVR, in various time intervals prior to sample collection were strongly associated with both $25(OH)D_3$ and $1,25(OH)_2D_3$ blood concentrations. We examined the association of ambient UVR in the month of blood collection and 1 month, 6 weeks and 2 months before blood collection, with both metabolites. The ambient UVR in 6 weeks before sample collection had the strongest magnitude of effect on both $25(OH)D_3$ and $1,25(OH)_2D_3$ (Table 2).

### Phenotypic and environmental factors

The associations between selected factors, based on their statistical significance and $r^2$, and vitamin D concentration ($25(OH)D_3$ and $1,25(OH)_2D_3$) in univariate analysis are presented in Table 2. We examined age, gender and anthropometrics measures as predictors of deseasonalised $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations. While

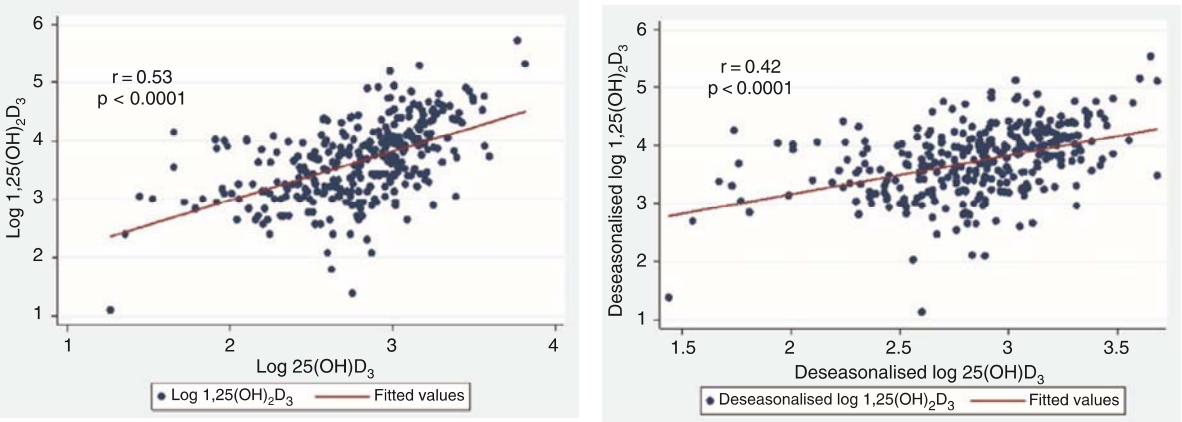


**Figure 1:** Comparison of serum deseasonalised 25(OH)$D_3$ and deseasonalised 1,25($OH_2D_3$ concentrations.
The correlation coefficient for the raw data r = 0.58, p < 0.0001.

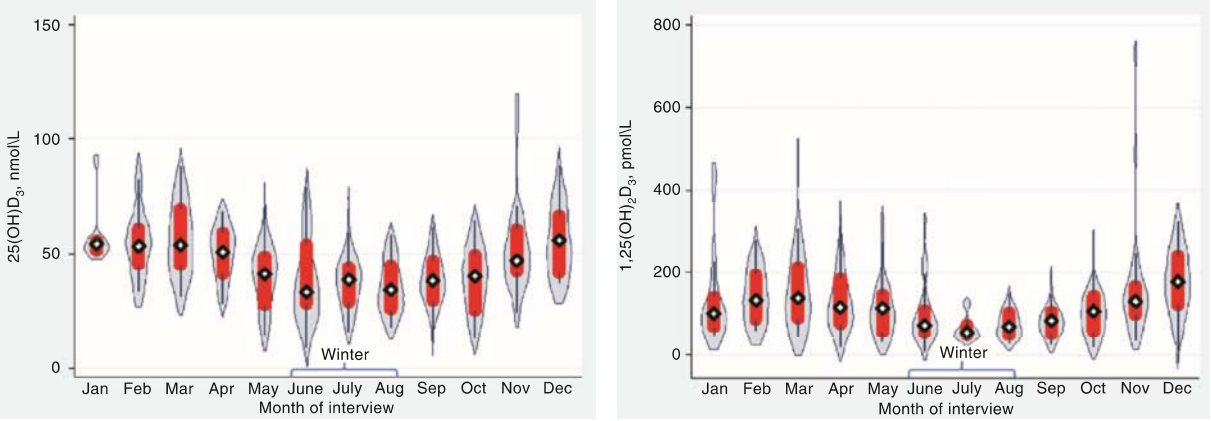


**Figure 2:** Month to month variation of serum 25(OH)$D_3$ and 1,25$(OH)_2D_3$ concentrations.
Summer (Dec to Feb), Autumn (Mar to May), Winter (Jun to Aug) and Spring (Sep to Nov).

there was a positive association between age and both deseasonalised 25(OH)$D_3$ and 1,25$(OH)_2D_3$ concentrations (p < 0.01 and p = 0.002, respectively), gender was not associated with them (p = 0.72 and p = 0.21, respectively). For anthropometric measures, height was the only factor associated with both 25(OH)$D_3$ and 1,25$(OH)_2D_3$ concentrations; mean increase of 0.03 units, p < 0.01 and 0.04 units, p = 0.01 per 10 cm increase, respectively. Weight was positively associated with 1,25$(OH)_2D_3$ (p = 0.04), and in contrast, age- and gender-adjusted BMI was inversely associated with 25(OH)$D_3$ (p < 0.01).

Caucasians had higher deseasonalised 25(OH)$D_3$ concentrations (mean difference 0.3, p < 0.001) but no difference in deseasonalised 1,25$(OH)_2D_3$ (p = 0.12) when compared to non-Caucasian. Lighter natural hair colour and skin colour were associated with higher deseasonalised 25(OH)$D_3$ but not with deseasonalised 1,25$(OH)_2D_3$. Lighter eye colour was associated with both deseasonalised 25(OH)$D_3$ and 1,25$(OH)_2D_3$ concentrations.

Child skin reaction during sun exposure, lifetime sun burns, freckling by the end of last summer and time in the sun during last summer and winter were associated with higher deseasonalised 25(OH)$D_3$ concentrations (Table 2). Only lifetime sun burns were associated with higher deseasonalised 1,25$(OH)_2D_3$ concentrations.

## Variation in 25(OH)$D_3$ and 1,25$(OH)_2D_3$ concentrations with genetic factors

Of the 12 selected SNPs, rs2282679, rs4588 (*GC*), rs6013897 (*CYP24A1*), rs12785878 (*DHCR7*), and rs10741657 (*CYP2R1*) were associated with higher concentrations of both deseasonalised 25(OH)$D_3$ and 1,25$(OH)_2D_3$ (data not shown). Complete genotyping data to calculate Metscore and

**Table 2:** The relationship of environmental and genetic factors with $25(OH)D_3$ and $1,25(OH)_2D_3$ in univariate analysis.

| Determinant | $Log_e$ deseasonalised $25(OH)D_3$ | | | $Log_e$ deseasonalised $1,25(OH)_2D_3$ | | |
|---|---|---|---|---|---|---|
| | Mean change[a] (95% CI) | p-Value | $(r^2)$[c] | Mean change[a] (95% CI) | p-Value | $(r^2)$[c] |
| Gender F (M) | −0.02 (−0.1, 0.07) | 0.72 | 0 | −0.08 (−0.22, 0.05) | 0.21 | 0 |
| Ethnicity | | | | | | |
| Caucasians/non-Caucasians | 0.30 (0.21, 0.40) | <0.001 | 13 | 0.13 (−0.03, 0.29) | 0.12 | 0 |
| Eye colour | | <0.001 | 11 | | 0.07 | 3 |
| Brown | Reference | | | Reference | | |
| Hazel | 0.12 (−0.01, 0.25) | 0.06 | | 0.18 (−0.03, 0.38) | 0.09 | |
| Green | 0.31 (0.15, 0.47) | <0.001 | | 0.14 (−0.11, 0.40) | 0.28 | |
| Blue/grey | 0.26 (0.15, 0.36) | <0.001 | | 0.20 (0.04, 0.37) | 0.02 | |
| Test of trend | | <0.01 | 96 | | 0.02 | 2.2 |
| Natural skin colour on examination | | <0.001 | 17 | | 0.61 | 1 |
| Dark | Reference | | | Reference | | |
| Olive | 0.48 (0.27, 0.69) | <0.001 | | 0.11 (−0.25, 0.46) | 0.56 | |
| Olive/medium | 0.55 (0.35, 0.75) | <0.001 | | 0.16 (−0.17, 0.50) | 0.34 | |
| Medium/fair | 0.65 (0.45, 0.85) | <0.001 | | 0.20 (−0.14, 0.53) | 0.25 | |
| Fair | 0.72 (0.50, 0.93) | <0.001 | | 0.24 (−0.11, 0.60) | 0.18 | |
| Test of trend | | <0.01 | 12.8 | | 0.11 | 1 |
| Weight (kg) | 0.001 (−0.01, 0.004) | 0.36 | 0.3 | 0.004 (0.00, 0.008) | 0.04 | 2 |
| Height (cm) | 0.003 (0.02, 0.89) | 0.002 | 4 | 0.004 (0.001, 0.01) | 0.01 | 2.3 |
| BMI (unit) | −0.01 (−0.01, 0.003) | 0.27 | 0.5 | 0.004 (−0.01, 0.02) | 0.61 | 0.1 |
| BMI category | | 0.002 | 4.2 | | 0.55 | 0.01 |
| Normal weight | Reference | | | Reference | | |
| Overweight | −0.20 (−0.32, −0.08) | 0.001 | | −0.10 (−0.30, 0.09) | 0.301 | |
| Obese | −0.14 (−0.26, −0.01) | 0.03 | | −0.06 (−0.25, 0.14) | 0.56 | |
| Test of trend | | <0.01 | 3.2 | | 0.42 | 0.3 |
| End of last summer-freckles | | <0.001 | 8 | | 0.05 | 2 |
| No freckles | Reference | <0.001 | | Reference | 0.14 | 2 |
| Few freckles | 0.20 (0.12, 0.29) | <0.001 | | 0.06 (−0.08, 0.20) | 0.40 | |
| Some freckles | 0.18 (0.03, 0.33) | 0.02 | | 0.27 (0.02, 0.52) | 0.04 | |
| Many freckles | 0.29 (0.03, 0.55) | 0.03 | | 0.26 (−0.16, 0.69) | 0.23 | |
| (Test of trend) | | <0.001 | 5.9 | | 0.03 | 1.5 |
| Lifetime sunburns | | <0.001 | 10 | | 0.05 | 2 |
| Never | Reference | | | Reference | | |
| Once | 0.22 (0.13, 0.32) | <0.001 | | 0.14 (−0.02, 0.30) | 0.08 | |
| 2–4 times | 0.26 (0.17, 0.36) | <0.001 | | 0.22 (0.06, 0.38) | 0.01 | |
| 5 or more times | 0.29 (0.00, 0.59) | 0.05 | | 0.17 (−0.31, 0.65) | 0.49 | |
| Test of trend | | <0.01 | 9 | | <0.01 | 2 |
| Time in sun in recent summer | 0.001 (0.00, 0.002) | 0.002 | 2.9 | 0.001 (−0.01, 0.002) | 0.26 | 0.4 |
| Time in sun in recent winter | 0.001 (0.0, 0.0014) | <0.01 | 2.2 | 0.001 (−0.01, 0.002) | 0.25 | 0.4 |
| Season of blood collection[d] | | <0.0001 | 13 | | <0.0001 | 0.14 |
| Winter | Reference | | | Reference | | |
| Summer | 0.46 (0.32, 0.61) | <0.001 | | 0.67 (0.44, 0.89) | <0.001 | |
| Autumn | 0.28 (0.16, 0.39) | <0.001 | | 0.52 (0.35, 0.70) | <0.001 | |
| Spring | 0.16 (0.05, 0.26) | <0.001 | | 0.36 (0.19, 0.53) | <0.001 | |
| Test of trend | | <0.01 | 5 | | 0.002 | 3 |
| UVR at month of interview[d] | 0.01 (0.008, 0.014) | <0.001 | 14 | 0.016 (0.01, 0.02) | <0.0001 | 12 |
| UVR 6 weeks before interview[d] | 0.01 (0.009, 0.02) | <0.001 | 18 | 0.017 (0.013, 0.02) | <0.0001 | 15 |
| Genetic factors | | | | | | |
| Metabolism score (rs2282679 + rs6013897) | | | | | | |
| 0 | Reference | | | Reference | | |
| 1 | −0.10 (−0.84, 0.65) | 0.80 | | −0.59 (−1.75, 0.57) | 0.32 | |
| 2 | −0.01 (−0.74, 0.72) | 0.99 | | −0.01 (−1.14, 1.13) | 0.99 | |
| 3 | 0.12 (−0.61, 0.85) | 0.75 | | 0.15 (−0.98, 1.29) | 0.79 | |
| 4 | 0.17 (−0.56, 0.90) | 0.64 | | 0.18 (−0.96, 1.32) | 0.76 | |
| Test of trend | | <0.001 | 4.4 | | <0.001 | 6.03 |

**Table 2** (continued)

| Determinant | $Log_e$ deseasonalised $25(OH)D_3$ | | | $Log_e$ deseasonalised $1,25(OH)_2D_3$ | | |
|---|---|---|---|---|---|---|
| | Mean change[a] (95% CI) | p-Value | ($r^2$)[c] | Mean change[a] (95% CI) | p-Value | ($r^2$)[c] |
| Synthesis score (rs12785878 + rs10741657) | | | | | | |
| 0 | Reference | | | Reference | | |
| 1 | 0.14 (−0.03, 0.31) | 0.10 | | 0.1 (−0.18, 0.38) | 0.48 | |
| 2 | 0.23 (0.07, 0.39) | 0.005 | | 0.15 (−0.12, 0.41) | 0.28 | |
| 3 | 0.38 (0.22, 0.55) | <0.001 | | 0.27 (0.00, 0.55) | 0.05 | |
| 4 | 0.33 (0.11, 0.56) | 0.004 | | 0.14 (−0.24, 0.51) | 0.46 | |
| Test of trend | | <0.001 | 7.79 | | 0.06 | 1 |

[a]Mean change of the natural-log-transformed deseasonalised $25(OH)D_3$ concentration per 1 unit change in exposure. [b]Mean change of the natural-log-transformed deseasonalised $1,25(OH)_2D_3$ concentration per 1 unit change in exposure. [c]Percentage of relative contribution. [d]Deseasonalised levels are not appropriate. This represents the mean change of the natural-log-transformed $25(OH)D_3$ and $1,25(OH)_2D_3$ for seasons per 1 category/unit change in exposure.

Synscore were available for 309 (96%) and 307 (95%) participants, respectively. While higher Metscore showed positive associations with both deseasonalised $25(OH)D_3$ and $1,25(OH)_2D_3$ ($p_{trend} < 0.001$: $r^2 = 0.04$ and $p_{trend} < 0.001$: $r^2 = 0.06$), Synscore showed a positive association with deseasonalised $25(OH)D_3$ ($p_{trend} < 0.001$: $r^2 = 0.08$) and a marginal association with deseasonalised $1,25(OH)_2D_3$ ($p_{trend} = 0.06$). Table 3 shows that in the 'genetic only' model, Synscore and Metscore were retained as major predictors for $25(OH)D_3$ with a contribution of 11.2% to its variance. Metscore was the only retained genetic predictor for $1,25(OH)_2D_3$ concentrations, with a contribution of 6.0% to its variance. No individual SNPs were predictive.

## Final predictive models for $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations

The final predictive model for $25(OH)D_3$ retained seven important factors: UVR 6 weeks before interview, ethnicity, skin colour, height, history of sun burn during life-time, Synscore and Metscore. Overall, these factors explained more than 43% of the variation in serum $25(OH)D_3$ concentrations. In contrast, the final predictive model for $1,25(OH)_2D_3$ retained four factors: UVR 6 weeks before interview, eye colour, height and Metscore. These factors explained over 25% of the variation of this biological active metabolite (Table 4).

Concentration $25(OH)D_3$ alone accounted for 18% of the variation in $1,25(OH)_2D_3$ concentrations. Interestingly, UVR 6 weeks before interview and Metscore remained positively associated with $1,25(OH)_2D_3$ even after further adjustment for $25(OH)D_3$ in the final model. No interaction was detected between the predictors of $1,25(OH)_2D_3$ in the final model. However, for $25(OH)D_3$ increasing Synscore had a greater impact on $25(OH)D_3$ concentrations among Caucasians than non-Caucasians.

# Discussion

This study demonstrates for the first time a multivariable model, which incorporates comprehensive environmental and genetic factors, for childhood circulating $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations. It shows the seasonal variation of $25(OH)D_3$ and vitamin D deficiency rate of approximately 80% in winter, based on $25(OH)D_3$ <20 ng/mL (<50 nmol/L), among this Australian paediatric population. Importantly, we are the first to report on the distribution of $1,25(OH)_2D_3$ among healthy children and its relation to a range of environmental and genetic factors. We found that the ambient UVR in 6 weeks before blood collection was the main factor influencing both child serum $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations. Ambient UVR 6 weeks before blood collection accounted for approximately 15% of $25(OH)D_3$ variance and 18% for $1,25(OH)_2D_3$ variance and contributed to a clear seasonal distribution for both with a late winter nadir. Not surprisingly in view of the endocrine regulation of 1 α-hydroxylase, the correlation between $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations was only moderate and we identified for the first time that selected gene variants related to vitamin D metabolism predicted higher $1,25(OH)_2D_3$ concentrations in children for a given $25(OH)D_3$ concentrations.

Strengths of this study include the detailed childhood sun exposure behaviours as well as extensive environmental data that enabled us to explore the interplay between genetic and environmental determinants. The availability of both $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations which was measured by a recommended assay in

**Table 3:** Multivariable linear regression models: environmental and genetic determinants of $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations.

| Determinant | $Log_e$ $25(OH)D_3$ | | | $Log_e$ $1,25(OH)_2D_3$ | | |
|---|---|---|---|---|---|---|
| | Mean change[a] (95%CI) | p-Value | $(r^2)$[c] | Mean change[b] (95%CI) | p-Value | $(r^2)$[c] |
| Environmental factors only | | | | | | |
| UVR 6 weeks before interview (per unit increase) | 0.011 (0.008, 0.014) | <0.001 | | 0.016 (0.01, 0.02) | <0.001 | |
| Lifetime sunburns[d] | 0.1 (0.05, 0.15) | <0.001 | | 0.11 (0.03, 0.2) | 0.01 | |
| End of summer freckles[d] | 0.07 (0.01, 0.13) | 0.02 | | | | |
| Time in sun during last winter [d] | 0.0007 (0.00, 0.001) | 0.02 | | | | |
| Environmental factors total contribution | | <0.001 | 27.89 | | <0.001 | 16.53 |
| Phenotypic factors only | | | | | | |
| Eye colour[d] | 0.043 (0.01, 0.08) | <0.03 | | 0.05 (0.001, 0.11) | 0.05 | |
| Natural skin colour[d] | 0.10 (0.06, 0.14) | <0.001 | | | | |
| Height (cm) | 0.003 (0.001, 0.005) | 0.002 | | | | |
| BMI Category[d] | −0.06 (−0.12, −0.01) | 0.03 | | | | |
| Phenotypic factors total contribution | | <0.001 | 20.95 | | <0.001 | 6.11 |
| Genetic factors only | | | | | | |
| Metabolism score[d] | 0.08 (0.04, 0.13) | <0.001 | | 0.17 (0.09, 0.24) | <0.001 | |
| Synthesis score[d] | 0.1 (0.06, 0.14) | <0.001 | | 0.06 (0.00, 0.13) | 0.06 | |
| Genetic factors total contribution | | <0.001 | 11.22 | | <0.001 | 6.03 |

[a]Mean change of the natural-log-transformed deseasonalised $25(OH)D_3$ concentration per 1 unit change in exposure. [b]Mean change of the natural-log-transformed deseasonalised $1,25(OH)_2D_3$ concentration per 1 unit change in exposure. [c]Percentage of relative contribution, [d]per category increase, see Table 2.

**Table 4:** Final multifactorial examination of $25(OH)D_3$ and $1,25(OH)_2D_3$ predictors in healthy Australian children.

| Determinant | $Log_e$ $25(OH)D_3$ | | | $Log_e$ $1,25(OH)_2D_3$ | | |
|---|---|---|---|---|---|---|
| | Mean change[a] (95%CI) | p-Value | $(r^2)$ | Mean change[b] (95%CI) | p-Value | $(r^2)$ |
| UVR 6 weeks before interview | 0.012 (0.009, 0.014) | <0.001 | | 0.02 (0.013, 0.023) | <0.001 | |
| Natural skin colour | 0.08 (0.04, 0.13) | <0.001 | | | | |
| Eye colour | | | | 0.07 (0.010, 0.13) | 0.02 | |
| Caucasian | 0.14 (0.04, 0.25) | 0.01 | | | | |
| Lifetime sunburns | 0.07 (0.02, 0.13) | <0.001 | | | | |
| Height (cm) | 0.002 (0.00, 0.004) | 0.03 | | 0.01 (0.001, 0.10) | 0.007 | |
| Metabolism score | 0.06 (0.01, 0.11) | 0.03 | | 0.13 (0.04, 0.21) | 0.005 | |
| Synthesis score | 0.07 (0.03, 0.12) | 0.002 | | | | |
| Final predictive model | | <0.001 | 43.54 | | <0.001 | 25.59 |

[a]Mean change of the natural-log-transformed deseasonalised $25(OH)D_3$ concentration per 1 unit change in exposure. [b]Mean change of the natural-log-transformed deseasonalised $1,25(OH)_2D_3$ concentration per 1 unit change in exposure.

a central laboratory [24], allowed us to conduct a comparison of their determinants which were rarely assessed in this age group. Because recent work in children has shown that $25(OH)D_3$ concentration may contain $D_3$ epimer (which has uncertain bioactivity), we provided fractionated $25(OH)D_3$ with $D_3$ epimer and $24,25(OH)_2D_3$ removed. Another strength is the use of the genetic data to construct a genetic score and its inclusion into the predictive models for both metabolites. Melbourne, Australia is well suited to this type of investigation as it is a city where vitamin D food fortification is uncommon and child supplementation is not routinely given. Despite a relatively large population, some of our findings were limited in power due to small numbers for particular genotypes and ethnic groups. Limitations of our study also include ethnicity was limited to parental report. However, it was consistent with the finding from the objective skin type assessment. Only a very basic dietary assessment was carried out and the use of vitamin D supplements in the last year was not recorded, (would be very low in our studied population as the major source of vitamin D was assumed to be sunlight exposure) [32]. In addition, our data did not include information about serum calcium, phosphate and parathyroid hormone concentrations which may contribute to the tight regulation of $1,25(OH)_2D_3$ concentrations [33, 34] via renal 1 $\alpha$-hydroxylation.

The finding that UVR levels at 6 weeks before interview had the strongest association with $25(OH)D_3$ was consistent with recent findings from Australia [17, 35] and Canada [36]. Previous studies have identified seasonal variation in $1,25(OH)_2D_3$ in geriatric participants and linked this to the seasonal variation in its substrate [$25(OH)D_3$] [37]. We are the first to show that UVR levels 6 weeks before interview remained a predictor of $1,25(OH)_2D_3$ even after adjustment for $25(OH)D_3$ concentrations. This finding, if replicated in future studies, could help explain why observational studies, often based on UVR-derived $25(OH)D_3$ concentrations in general populations, have shown that higher $25(OH)D_3$ concentrations were associated with reduced disease risk compared with randomised control trials, using dietary vitamin $D_3$ supplementation have not [38], i.e. in the former, $25(OH)D_3$ concentrations are a marker of past UVR exposure and our data indicate this would have been with a hidden additional benefit on $1,25(OH_2)D_3$ concentrations and thus presumably active vitamin D activity. In contrast, higher $25(OH)D_3$ concentrations following vitamin $D_3$ dietary supplementation would not be accompanied by any UVR-induced boost to $1,25(OH)_2D_3$ [39].

Most previous studies showed a consistent effect of *CYP2R1* (rs10741657) [16, 40, 41], *DHCR7* (rs12785878) [16, 42], *CYP24A1* (rs6013897) [16, 40] and *GC* (rs2282679) [16, 40, 41] which was found to be associated with vitamin D concentrations in our study. *GC*-globulin is the major VDBP which acts as a transporter for both $25(OH)D_3$ and $1,25(OH)_2D_3$ leaving <0.1% and <1%, respectively, to circulate in free form [43, 44]. Lower VDBP, which is linked to *GC* variants, were previously reported to be associated with low $25(OH)D_3$ concentrations [16]. Combining *GC* variants with *CYP24A1* (rs6013897) on Metscore provided a more powerful instrument for the prediction of $25(OH)D_3$ concentrations in our study which is consistent with a previous report [29]. Interestingly, we extend this concept to the association of Metscore with $1,25(OH)_2D_3$ concentrations and showed the same pattern. This suggests that bioavailability of both metabolites are affected by these gene variants.

Although the final predictive models demonstrated here were only able to account for 43% and 25% of $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations, respectively. This compares well with the 1%–4% accounted for by genetic factors in a vitamin D GWAS study [16]. Previously, a vitamin D synthesis or metabolism score has been successfully used as an instrumental variable in a Mendelian randomisation study [28]. Here we confirm that such scores have substantially improved the predictive model for $25(OH)D_3$ concentrations and we extended this approach by showing that the Metscore predicted $1,25(OH)_2D_3$ concentrations, beyond the blood concentrations of $25(OH)D_3$.

## Conclusions

Here, we have developed predictive models for both $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations in a population of Australian children. These findings are based on a multiethnic population and can be generalised to healthy child populations. The findings highlight the importance of both environmental factors, in particular ambient UVR levels 6 weeks before blood collection, and genetic factors in determining both $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations in children.

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